

## Interactive Effects of Polylactic Acid with Different Aluminum Concentrations on Growth, Pigment Concentrations, and Carbohydrate Accumulation of *Azolla*

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**ABSTRACT.**—The interactive effects of  $1 \mu\text{g l}^{-1}$  polylactic acid (PLA) with aluminum concentrations of 2, 4, 8, and  $16 \text{ mg l}^{-1}$  on *Azolla caroliniana* growth, chlorophyll, carotenoid, anthocyanin, and carbohydrate accumulation were examined. *Azolla* growth was decreased with increases in aluminum concentration. Furthermore, the growth reduction was less pronounced when PLA was present. Chlorophyll *a* and *b* concentrations were decreased in plants grown at Al concentrations of  $4 \text{ mg l}^{-1}$  and higher. Polylactic acid reduced the influence of Al concentrations of 4, 8, and  $16 \text{ mg l}^{-1}$  on chlorophyll *a* and *b*. Similarly, carotenoid concentrations were decreased as Al concentrations increased in the growth media. Anthocyanin concentration was increased at  $4.0 \text{ mg l}^{-1}$  Al without PLA and at higher Al concentrations with or without PLA. In general, carbohydrate accumulation increased as Al concentration increased. However, this increase in carbohydrates was less pronounced in the presence of PLA. In the absence of Al from the growth media, treatment with  $1 \mu\text{g l}^{-1}$  PLA resulted in an increase in plant growth, chlorophyll, and carotenoid concentration and a decrease in carbohydrate accumulation.

Aluminum (Al) toxicity is an important component of the damaging effects of acid rain in rivers and lakes. Rueter et al. (1987) reported that acid rain not only increased total soluble Al concentration, but also changed the affinity of organic and inorganic complexes with metals. Sprenger and McIntosh (1989) showed that Al concentrations were elevated in both water and submerged aquatic macrophytes (*Ranunculus ololeucos* J. Lloyd, *Luronium natans* Raf.) in acidified waters. According to Stanley (1974), an addition of  $92 \mu\text{mol l}^{-1}$  Al to well-buffered medium inhibited 50% of the root growth of *Myriophyllum spicatum* L.

The toxicity of Al depends upon a number of factors, including pH and organic matter content (Tan and Binger, 1986). Aluminum toxicity increases at a pH below 5 because its solubility increases considerably. Of the different species of Al,  $\text{Al}^{3+}$  is the only significant one that is found in freshwater at a pH of 4 or less (Poleo, 1995). In general, Al has been shown to interfere with cell division, decrease respiration rates, interfere with enzymes governing the deposition of polysaccharide in cell walls, increase cell wall rigidity, and decrease the uptake and transport of water and several elements including Ca, Mg, P, and K in plants (Foy et al., 1978).

The earliest recorded use of lactic acid as a plant growth regulator was by

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Blumenthal and Meyer (1924), who observed callus formation from carrot slices exposed to lactic acid. In response to growth-inhibitory levels of heavy metals, plants synthesize metal-binding phytochelatins to detoxify excess metals (Kinnersley, 1993). Biostimulants such as polylactic acid have properties in common with phytochelatins and could be employed as a heavy metal detoxifier (Kinnersley, 1993). Aqueous lactic acid contains polymers of lactic acid which promote growth in some plants and unicellular algae (Kinnersley, 1993). This study was designed to evaluate the interactive effects of polylactic acid ( $1 \mu\text{g l}^{-1}$ ) with different aluminum concentrations (2, 4, 8, and  $16 \text{ mg l}^{-1}$ ) on *Azolla* growth, chlorophyll, carotenoid and anthocyanin content and carbohydrate concentration.

#### MATERIALS AND METHODS

Cultures of *Azolla caroliniana* Willd. were grown in a diluted (1:40) Hoagland solution (Hoagland and Arnon, 1938) at pH 6.0. Plants were placed in a growth chamber with a 14 h photoperiod at  $220 \mu\text{mol m}^{-2} \text{ s}^{-1}$  photosynthetic photon flux density, and temperatures of  $25 \pm 1^\circ\text{C}$ . A total of 2400 plants was selected arbitrarily and placed into 250 ml Erlenmeyer flasks (12 flasks per treatment). The flasks contained 125 ml diluted (1:40) Hoagland solution (pH of 3.8), and received 20 plants. The treatments were Al concentrations of 0, 2, 4, 8, and  $16 \text{ mg l}^{-1}$  with and without the presence of  $1 \mu\text{g l}^{-1}$  dissolved polymer L-lactic acid. Polylactic acid was prepared following the method of Kinnersley et al. (1990). Aluminum was supplied in the form of  $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ . The concentration of PLA was selected based on the earlier finding that the highest *Azolla* growth was obtained at PLA concentration of  $1 \mu\text{g l}^{-1}$  (Ayala-Silva and Al-Hamdani, 1997). *Azolla* was grown at the above conditions for 14 days and the growth medium was changed at the end of the first week. At the end of the second week of treatment, the plants from six randomly selected flasks of each treatment were used for dry weight and nonstructural carbohydrates measurements. The plants in the remaining six flasks of each treatment were used for chlorophyll *a* and *b*, carotenoid and anthocyanin determinations.

**DRY WEIGHT.**—Samples were oven dried for 48 h at  $70^\circ\text{C}$  and the dry weight was recorded. The dried samples were then stored at  $-20^\circ\text{C}$  for subsequent carbohydrate analysis.

**CHLOROPHYLL/CAROTENOID CONTENT.**—Each sample (0.10 g fresh weight) was placed in a 10 ml vial (6 per treatment) containing 5 ml of N,N-dimethylformamide (N,N-DMF). The samples were stored in the dark at  $4^\circ\text{C}$  for 36 h and assayed spectrophotometrically following the procedures of Inskeep and Bloom (1985). Total carotenoid concentration was determined spectrophotometrically from the N,N-DMF extraction, and total concentration was calculated using the formula of Doong et al. (1993).

**ANTHOCYANIN CONTENT.**—Samples of 0.10 g fresh weight were homogenized in 5 ml of methanol containing 1% HCl (v/v). The homogenates were then

TABLE 1. Interactive effects of polylactic acid (PLA) concentration of  $1 \mu\text{g l}^{-1}$  and different concentrations of aluminum ( $\text{mg l}^{-1}$ ) on *Azolla* growth. LSD = Least Significant Difference value for difference between means within a column.

Treatment	Plant—Dry wt. (mg)
Control (without Al and PLA)	34.0
PLA	41.0
2.0 Al with PLA	30.0
2.0 Al without PLA	27.0
4.0 Al with PLA	25.0
4.0 Al without PLA	23.0
8.0 Al with PLA	25.0
8.0 Al without PLA	21.0
16.0 Al with PLA	17.0
16.0 Al without PLA	14.0
LSD (0.05)	4.0
LSD (0.01)	5.0

filtered and absorbance of the extracts determined spectrophotometrically by the method of Mancinelli (1990).

**CARBOHYDRATE DETERMINATION.**—Carbohydrate analysis of the plant samples was conducted following a procedure slightly modified from that outlined by Chatterton et al. (1987). Samples were ground into a fine powder and a 100–500 mg portion was placed in a sealed vial and used for the determination of soluble sugars, starch, and total nonstructural carbohydrates (TNC) as reported in detail by Wilson and Al-Hamdani (1997).

**STATISTICAL ANALYSIS.**—This experiment was repeated twice and analyzed statistically as a randomized complete block design (Steel and Torrie 1980). This design ensured that observed differences in plant performances were largely due to treatments rather than variation among blocks (experiment conducted at different times). Mean separations for the treatments that had significant F values ( $P > 0.05$ ) in the ANOVA tests were on the basis of the least significant difference (LSD) test (Steel and Torrie, 1980).

## RESULTS AND DISCUSSION

After, 14 days, growth of *Azolla* was decreased as the aluminum concentrations were increased. The exception was that of  $2 \text{ mg l}^{-1}$  Al, which in the presence of PLA did not significantly decrease *Azolla* growth (Table 1). The highest growth reduction (58.8%) was documented for plants grown at  $16.0 \text{ mg l}^{-1}$  Al in the absence of PLA (Table 1). Aluminum was shown to interfere with cell division, decrease respiration rates, interfere with enzymes governing the deposition of polysaccharide in cell walls, increase cell wall rigidity, and decrease the uptake, transport, and use of water and several elements including Ca, Mg, P, and K in plants (Foy et al., 1978). The presence of PLA in the media had no significant influence on Al effects on plant growth at the ex-

TABLE 2. Interactive effects of polylactic acid (PLA) concentration of  $1 \mu\text{g l}^{-1}$  and different concentrations of aluminum ( $\text{mg l}^{-1}$ ) on chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), carotenoid and anthocyanin concentration in *Azolla*. LSD = Least Significant Difference value for difference between means within a column.

Treatment	Chl <i>a</i> $\text{mg g}^{-1}$ fr. wt	Chl <i>b</i> $\mu\text{g g}^{-1}$ fr. wt	Carotenoid	Anthocyanin
Control (without Al and PLA)	7.53	5.49	72.39	2.00
PLA	8.32	6.15	81.69	2.84
2.0 Al with PLA	7.42	5.29	65.52	2.90
2.0 Al without PLA	7.40	5.14	62.08	2.93
4.0 Al with PLA	6.31	4.40	64.12	3.03
4.0 Al without PLA	5.81	3.98	63.81	3.72
8.0 Al with PLA	3.17	2.13	37.44	11.46
8.0 Al without PLA	2.47	1.34	35.40	11.90
16.0 Al with PLA	2.76	2.14	32.30	17.28
16.0 Al without PLA	2.35	1.42	30.33	19.57
LSD (0.05)	4.00	3.40	5.89	1.42
LSD (0.01)	5.30	4.60	7.89	1.90

aminated Al concentrations (Table 1). However, in the absence of Al, PLA caused significant increase in *Azolla* growth. Furthermore, the reduction of growth as influenced by Al concentrations was less pronounced when PLA was present. Aqueous lactic acid containing polymers of lactic acid was reported to promote growth in a variety of plants and unicellular algae (Kinnersley, 1993). In earlier studies, Kinnersley et al. (1990) found that  $1000 \mu\text{g l}^{-1}$  of the polymer L-lactic acid resulted in a significant increase plant biomass in duckweed (*Lemna minor* L.), an effect that was accompanied by an increase in chlorophyll accumulation.

Chlorophyll *a* and *b* concentrations were decreased in plants grown at Al concentration of  $4 \text{ mg l}^{-1}$  and higher (Table 2). In the absence of Al from the growth medium, PLA increased the concentration of chlorophylls *a* and *b* (Table 2). Similarly, the concentration of carotenoid in *Azolla* was increased by the presence of  $1 \mu\text{g l}^{-1}$  PLA and decreased as Al concentrations increased in the growth media (Table 2).

The actual mechanism of action of polylactic acid is unknown. However, the increase in plant growth and chlorophyll concentration documented here for *Azolla* could be due to polylactic acid enhancing plant nutrient assimilation or utilization. Kinnersley et al. (1990) found that polylactic acid stimulated growth of *Lemna* in reduced nutrient media. On the other hand, the interactions of Al with mineral nutrients have been well-documented in many plant systems in the recent past. For example, Al has been shown to interfere with the uptake and accumulation of certain nutrients and the transport and use of essential elements, including N, P, K, Ca, Mg, Na, Fe, Cu, Mn, and Zn (Balakumar et al., 1991). In response to elevated levels of heavy metals, plants synthesize metal-binding phytochelatins to detoxify excess metals. Biostimulants such as polylactic acid have properties similar to phytochelatins and could be employed as a heavy metal detoxifiers (Kinnersley, 1993).

TABLE 3. Interactive effects of polylactic acid (PLA) concentration of  $\mu\text{g l}^{-1}$  and different concentrations of aluminum ( $\text{mg l}^{-1}$ ) on carbohydrate concentration in *Azolla*. LSD = Least Significant Difference value for difference between means within a column.

Treatment	Sucrose	Starch	Total nonstructural carbohydrates
	----- ( $\mu\text{g g}^{-1}$ dry wt.) -----		
Control (without Al and PLA)	5.48	261.42	355.50
PLA	6.80	217.50	300.85
2.0 Al with PLA	7.17	292.78	395.37
2.0 Al without PLA	8.41	314.50	415.46
4.0 Al with PLA	9.51	322.13	414.20
4.0 Al without PLA	10.90	393.46	499.17
8.0 Al with PLA	11.72	362.00	469.70
8.0 Al without PLA	12.30	400.00	510.03
16.0 Al with PLA	13.10	477.00	590.97
16.0 Al without PLA	17.90	515.00	634.47
LSD (0.05)	1.38	37.46	39.58
LSD (0.01)	1.84	49.47	52.79

Anthocyanin concentration was increased at the Al concentration of  $4.0 \text{ mg l}^{-1}$  without the presence of PLA and at higher Al concentrations with or without PLA (Table 2). The highest increase in anthocyanin concentration (879%) was obtained for plants grown at  $16 \text{ mg l}^{-1}$  Al without PLA. Anthocyanin induction in *Azolla* has not been yet extensively studied. These measurements may provide an opportunity to relate its formation to biomass and chlorophyll formation. Many aquatic plants possess anthocyanins. These pigments are produced in response to various stress-related factors such as high light intensity, high temperatures or nutritional limitations (Doong et al., 1993). Haug (1984) suggested that anthocyanins are capable of chelating Al. This indicates that *Azolla* increased anthocyanin synthesis as mechanism of detoxifying Al.

Sucrose concentration of *Azolla* increased with the increase in Al concentration in the growth media (Table 3). However, sucrose concentration was not significantly different among the same Al concentrations when PLA was present. The exception was for plants grown at  $16 \text{ mg l}^{-1}$  Al, in which the concentration of sucrose was increased by 36.6% in comparison to that of the same concentration in the absence of PLA. Starch accumulation in *Azolla* was increased as Al concentrations were increased in the growth media (Table 3). However, this increase in starch accumulation was less distinct in the presence of PLA. In addition, PLA influenced reduction in starch accumulation in plants grown at Al concentrations greater than  $2 \text{ mg l}^{-1}$  in comparison to that obtained of plants grown at the same Al concentrations in the absence of PLA. Similar results were obtained for the TNC with the exception that in the absence of Al, PLA resulted in an decrease in total nonstructural carbohydrates (TNC) accumulation (Table 3). This should not be interpreted as Al causing an increase in carbohydrate synthesis. Rather, it most likely resulted from a reduction in carbohydrate utilization. An increase in carbohydrate accumulation in response to Al was also found to take place in *Salvinia minima* Baker

(Gardner and Al-Hamdani, 1997). Schroll (1978) observed that metal toxicity inhibited photosynthesis less than overall plant growth. Furthermore, Roy et al. (1988) reported that Al decreased respiration and carbohydrate utilization. Lower rates of carbon assimilation and a decrease in yield are associated with carbohydrate accumulation in several plant species (Azcon-Bieto 1983). It seems likely, therefore, that Al caused an increase in carbohydrate accumulation as plant growth was decreased.

In conclusion, this study has shown that Al is toxic to *Azolla* plants when provided at concentrations higher than  $2 \text{ mg l}^{-1}$ . In most cases, polylactic acid reduced the damaging effects of aluminum. This may be due to binding of polylactic acid with Al, resulting in a lower effective Al concentration within plant cells.

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